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## Original Contribution

Genetic ablation of phagocytic NADPH oxidase in mice limits TNF $\alpha$ -induced inflammation in the lungs but not other tissuesWei-Jian Zhang<sup>a,b,\*</sup>, Hao Wei<sup>a</sup>, Ying-Tzang Tien<sup>c</sup>, Balz Frei<sup>a,b,\*</sup><sup>a</sup> Linus Pauling Institute, Oregon State University, Corvallis, OR 97331, USA<sup>b</sup> Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331, USA<sup>c</sup> Histopathology Laboratory, School of Medicine, University of Washington, Seattle, WA 98195, USA

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## ABSTRACT

In vitro and limited in vivo evidence suggests that reactive oxygen species derived from NADPH oxidases (NOX-ROS) play an important role in inflammatory responses by enhancing the activity of redox-sensitive cell signaling pathways and transcription factors. Here, we investigated the role of NOX-ROS in TNF $\alpha$ -induced acute inflammatory responses in vivo, using mice deficient in the gp91<sup>phox</sup> (NOX2) or p47<sup>phox</sup> subunits of NADPH oxidase. Age- and body weight-matched C57BL/6J wild-type (WT) and gp91<sup>phox</sup> or p47<sup>phox</sup> knockout mice were injected intraperitoneally with 50  $\mu$ g TNF $\alpha$ /kg bw or saline vehicle control and sacrificed at various time points up to 24 h. Compared to WT mice, gp91<sup>phox</sup> <sup>-/-</sup> mice exhibited significantly diminished ( $P < 0.05$ ) TNF $\alpha$ -induced acute inflammatory responses in the lungs but not other tissues, including heart, liver, and kidney, as evidenced by decreased activation of the redox-sensitive transcription factor NF- $\kappa$ B, and decreased gene expression of interleukin (IL)-1 $\beta$ , IL-6, TNF $\alpha$ , E-selectin, and other cellular adhesion molecules. Similar results were observed in p47<sup>phox</sup> <sup>-/-</sup> mice. Interestingly, decreased lung inflammation in knockout mice was accompanied by increased leukocyte infiltration into the lungs compared to other tissues. Our data suggest that phagocytic NOX-ROS signaling plays a critical role in promoting TNF $\alpha$ -induced, NF- $\kappa$ B-dependent acute inflammatory responses and tissue injury specifically in the lungs, which is effected by preferential leukocyte infiltration.

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The NOX family of NADPH oxidases, a highly regulated, membrane-bound group of enzyme complexes found in a variety of phagocytic and nonphagocytic cells, is a major biological source of superoxide radicals and hydrogen peroxide [1]. In phagocytic cells the core enzyme consists of five subunits: p40<sup>phox</sup>, p47<sup>phox</sup>, p67<sup>phox</sup>, p22<sup>phox</sup>, and gp91<sup>phox</sup> (NOX2). In addition, two low-molecular-weight GTP-binding proteins, Rap 1A and Rac1/2, are involved in activation of the enzyme. Mice that are genetically deficient in gp91<sup>phox</sup> or p47<sup>phox</sup> exhibit a reduced capacity to generate superoxide radicals [2–4].

Activation of NADPH oxidases takes place either at the plasma membrane, leading to release of superoxide radicals into the extracellular environment, or at intracellular membranes, leading to local

formation of superoxide radicals and hydrogen peroxide in intracellular compartments. Phagocytic NADPH oxidase, originally described in neutrophils and macrophages, serves a critical function in host defense against invading pathogenic microorganisms. The nonphagocytic NADPH oxidases, found in smooth muscle cells, synovial cells, chondrocytes, endothelial cells, and epithelial cells, produce smaller amounts of reactive oxygen species (ROS), which may play a critical role in cell signaling and normal cell growth and function [1]. However, the mechanisms underlying intracellular ROS production and the relative concentrations and activities of NADPH oxidases in each of the nonphagocytic cell types have not been fully determined.

In recent years there has been increasing evidence indicating that ROS, and in particular hydrogen peroxide, act as mediators in cell signaling in a broad array of physiological and pathophysiological responses [1,5,6]. The regulation of gene transcription of proinflammatory cytokines, chemokines, and cellular adhesion molecules has been shown to be mediated by reduction-oxidation (redox)-sensitive signaling pathways and transcription factors, such as nuclear factor  $\kappa$ B (NF- $\kappa$ B) and activator protein-1 (AP-1) [7,8]. NF- $\kappa$ B activation leads to upregulation of a wide array of inflammatory genes critical for neutrophilic inflammation and host defense against infectious agents and may also cause the systemic inflammatory response syndrome [9,10]. A variety of in vitro studies have suggested that ROS act as second messengers affecting NF- $\kappa$ B activation [11–13]. Thus, it is possible that

**Abbreviations:** AP-1, activator protein-1; BAL, bronchoalveolar lavage; bw, body weight; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICAM-1, intercellular adhesion molecule-1; IKK, I $\kappa$ B kinase; IL, interleukin; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; MPO, myeloperoxidase; NF- $\kappa$ B, nuclear factor  $\kappa$ B; NOX-ROS, NADPH oxidase-derived ROS; PMN, polymorphonuclear neutrophil; redox, reduction-oxidation; ROS, reactive oxygen species; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; VCAM-1, vascular cell adhesion molecule-1; WT, wild type.

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NADPH oxidase-derived ROS (NOX-ROS) play an important role in regulating cell signaling and host inflammatory responses in both phagocytic and nonphagocytic cells. However, there is limited and controversial evidence from *in vivo* studies to support this notion [3,4,14–21].

Tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) is a major mediator of inflammation, immunity, and apoptosis and has been implicated in the pathogenesis of a wide spectrum of human diseases [22,23]. It promotes inflammation by increasing blood neutrophil concentrations, enhancing expression of multiple inflammatory mediators, and inducing adhesion of neutrophils and monocytes to vascular endothelium and their subsequent tissue infiltration. Through binding to its cellular receptors, TNF $\alpha$  activates multiple signaling pathways [23,24]; ROS have been implicated in mediating TNF $\alpha$  signaling, although their source has not been fully established [5]. Recent evidence indicates that NOX-ROS contribute to TNF $\alpha$ -induced NF- $\kappa$ B activation and adhesion molecule and IL-8 expression in cultured endothelial cells [14,25–28]. However, the role of NOX-ROS in cell signaling and gene expression in TNF $\alpha$ -induced acute inflammatory responses *in vivo* remain to be fully determined.

We have recently shown that genetic deficiency of gp91<sup>phox</sup> or p47<sup>phox</sup> does not diminish, but rather enhances, lipopolysaccharide (LPS)-induced acute inflammatory responses *in vivo*, suggesting that NOX-ROS do not play a role in enhancing inflammation, but rather in its resolution [21]. As the signaling pathways in TNF $\alpha$ -induced acute inflammation are distinct from those triggered by LPS, in this study we investigated whether limiting NOX-ROS production by genetic deficiency of gp91<sup>phox</sup> or p47<sup>phox</sup> is associated with impairment or enhancement of TNF $\alpha$ -induced acute inflammatory responses *in vivo*.

## Materials and methods

### Animals and experimental procedures

Age- and body weight-matched female C57BL/6J WT, gp91<sup>phox</sup> <sup>-/-</sup>, and p47<sup>phox</sup> <sup>-/-</sup> mice at 10–12 weeks of age and weighing 20–22 g were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed under specific-pathogen-free conditions and in a temperature- and humidity-controlled environment (12-h light/dark cycle) with unlimited access to tap water and food. Animals were acclimated for 7–10 days before initiation of experiments. All animal procedures were reviewed and approved by the Oregon State University Institutional Animal Care and Use Committee.

Wild-type, gp91<sup>phox</sup> <sup>-/-</sup>, and p47<sup>phox</sup> <sup>-/-</sup> mice were randomly assigned to receive intraperitoneal (ip) injection of the vehicle, Hanks' balanced salt solution (HBSS; Sigma–Aldrich, St. Louis, MO, USA), as controls, or 50  $\mu$ g of murine recombinant TNF $\alpha$  (R&D Systems, Minneapolis, MN, USA) per kilogram body weight (bw) and sacrificed 1, 3, 8, or 24 h after injection. Blood and various tissues were collected for further analysis.

### Serum concentrations of inflammatory mediators

Serum concentrations of soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1), monocyte chemoattractant protein-1 (MCP-1), and TNF $\alpha$  were measured by quantitative colorimetric ELISA kits from R&D Systems. The reported sensitivities of the assays are 5 pg/ml sVCAM-1, 29 pg/ml sICAM-1, 2 pg/ml MCP-1, and 5 pg/ml TNF $\alpha$ .

### Tissue mRNA levels of inflammatory mediators

Total RNA was isolated from various tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was performed using the high-capacity cDNA archive kit from Applied Biosystems (Foster City, CA, USA). mRNA levels of E-selectin, VCAM-1, ICAM-1, MCP-1, TNF $\alpha$ , IL-1 $\beta$ , IL-6, myeloperoxidase (MPO), and glyceraldehyde-3-

phosphate dehydrogenase (GAPDH) were quantitated by real-time PCR. All primers and probes were purchased as kits from Applied Biosystems. The assays are supplied as a 20 $\times$  mixture of PCR primers and TaqMan minor-groove binder 6-FAM dye-labeled probes with a nonfluorescent quencher at the 3' end. TaqMan quantitative PCR (40 cycles of 95  $^{\circ}$ C for 15 s and 60  $^{\circ}$ C for 1 min) was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) in 96-well plates with the ABI Prism 7500 sequence detection system (Applied Biosystems). To obtain relative quantitation, two standard curves were constructed in each plate with one target gene and an internal control gene (GAPDH). Standard curves were generated by plotting the threshold cycle number values against the log of the amount of input cDNA and used to quantitate the expression of the various target genes and GAPDH gene in the same sample.

### Bronchoalveolar lavage

Mice were sacrificed 8 h after HBSS (control) or TNF $\alpha$  injection. The left lung was tied off, and the right lung was lavaged by intratracheal injection of two sequential 0.6-ml aliquots of HBSS, followed by aspiration. Recovered fluid was pooled and centrifuged. Supernatants were preserved for measurement of total albumin concentration using an enzyme-linked immunoassay (Bethyl Labs, Montgomery, TX, USA). The leukocyte pellet was resuspended in HBSS and cell numbers were counted using a hemocytometer.

### Preparation and morphological evaluation of lungs

Mice were sacrificed 8 h after HBSS (control) or TNF $\alpha$  injection. The left lung was removed and fixed with 10% neutral-buffered formalin and embedded in paraffin. After dewaxing and rehydration, 5- $\mu$ m-thick paraffin sections were stained with hematoxylin and eosin. Neutrophils were counted from 10 randomly selected fields (20 $\times$  magnification, 0.4 $\times$ 0.4 mm<sup>2</sup>) in each section. A total of 30 fields were screened from sections selected from the upper, middle, and lower parts of the left lung.

### Western blots

Lung tissue samples were homogenized in 1 ml lysis buffer (Cell Signaling Technology, Danvers, MA, USA) followed by centrifugation at 14,000g and 4  $^{\circ}$ C for 15 min. The supernatant containing solubilized proteins was recovered, frozen in aliquots, and stored at  $-80^{\circ}$  C until analysis. Protein samples were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated overnight with mouse anti-MPO antibody (R&D Systems) or rabbit anti- $\alpha$ -tubulin antibody (Cell Signaling), after being blocked with 0.1% Tween 20 in Tris-buffered saline containing 5% skim milk powder. The membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat anti-rabbit antibody (Cell Signaling) for 1 h at room temperature. The proteins were detected by enhanced chemiluminescence (ECL; GE Healthcare, Buckinghamshire, UK).

### Lung myeloperoxidase activity

Lung tissue samples were homogenized in 50 mM phosphate buffer (pH 7.4) and centrifuged at 10,000g for 10 min at 4  $^{\circ}$ C, and the resulting pellet was resuspended in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma–Aldrich). The suspension was subjected to three cycles of freezing and thawing and disrupted further by sonication (40 s). Subsequently, the samples were centrifuged at 10,000g for 10 min at 4  $^{\circ}$ C, and the supernatant was used for measuring MPO activity with the Myeloperoxidase Activity Assay Kit from Northwest Life Science Specialties (Vancouver, WA, USA). One unit of MPO activity is defined as the

amount of enzyme that catalyzes the formation of 1 nM taurine chloramine from taurine at pH 6.5, 25 °C, over 30 min in the presence of 100 mM chloride and 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>. The MPO activity was normalized to the total protein concentration.

#### Activation of nuclear transcription factors

Nuclear extracts were prepared from various tissues using nuclear extract kits (Active Motif, Carlsbad, CA, USA). For analysis of activation of nuclear transcription factors, ELISA-based assays (Active Motif) were used to determine the DNA binding activity of NF- $\kappa$ B (p65) and AP-1 (c-fos). The specificity of binding was confirmed by competition with either wild-type or mutant oligonucleotides. The wild-type oligonucleotides prevent NF- $\kappa$ B and AP-1 binding to the probe immobilized on the plates. Conversely, the mutated oligonucleotides have no effect on NF- $\kappa$ B and AP-1 binding activity.

#### Statistical analysis

The data were calculated as means  $\pm$  SEM and analyzed by unpaired Student's *t* test or ANOVA followed by Holm–Sidak post hoc test. Statistical significance was set at *P* < 0.05.

### Results

#### Genetic deficiency of gp91<sup>phox</sup> or p47<sup>phox</sup> does not diminish TNF $\alpha$ -induced increases in serum concentrations of inflammatory mediators

To assess the time course of TNF $\alpha$ -induced acute systemic inflammation in mice, serum levels of selected inflammatory mediators were measured up to 24 h after ip injection of TNF $\alpha$  (50  $\mu$ g/kg bw). As shown in Fig. 1, TNF $\alpha$  treatment caused a rapid increase in serum concentrations of sVCAM-1, sICAM-1, MCP-1, and TNF $\alpha$ : TNF $\alpha$  peaked at 1 h, and the other inflammatory mediators were significantly increased at 3 h. sVCAM-1 and sICAM-1 remained elevated up to 24 h, whereas MCP-1 and TNF $\alpha$  returned to baseline at 8 h. Interestingly, these TNF $\alpha$ -induced changes in serum levels of inflammatory mediators were indistinguishable between gp91<sup>phox</sup> <sup>-/-</sup> and WT mice at all time

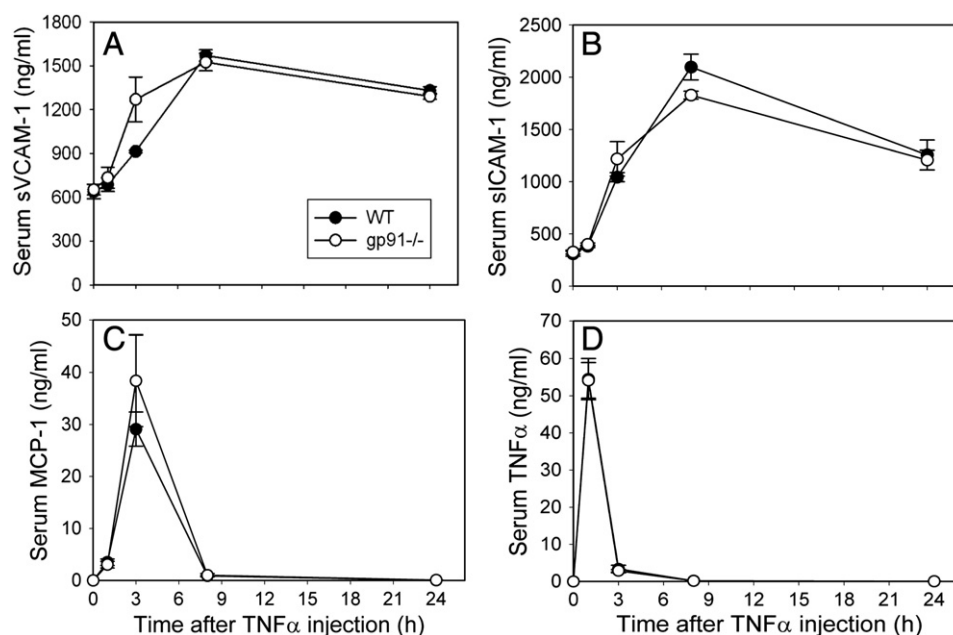
points examined (Fig. 1). Similar results were observed in serum of p47<sup>phox</sup> <sup>-/-</sup> mice 3 h after TNF $\alpha$  injection (data not shown). These data suggest that NADPH oxidase activity is not required for TNF $\alpha$ -induced systemic inflammatory responses.

#### Genetic deficiency of gp91<sup>phox</sup> or p47<sup>phox</sup> significantly diminishes TNF $\alpha$ -induced upregulation of inflammatory gene expression in the lungs but not other tissues

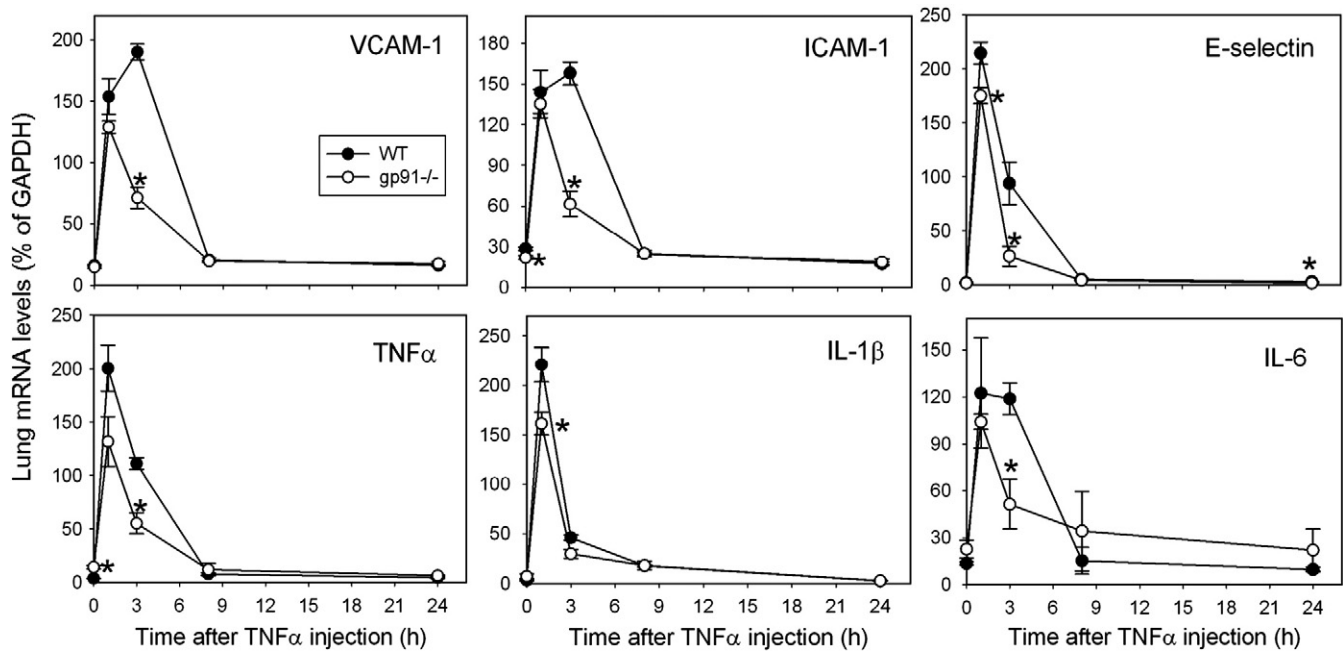
To assess TNF $\alpha$ -induced gene expression of inflammatory mediators, their mRNA levels were measured in lung, heart, liver, kidney, small intestine, and spleen. As expected, lung mRNA levels of all inflammatory mediators increased in a time-dependent manner after TNF $\alpha$  administration to WT mice (Fig. 2). VCAM-1, ICAM-1, and IL-6 mRNA levels were increased substantially 1 h after TNF $\alpha$  injection and remained elevated for at least 2 h before declining to baseline after 8 h. E-selectin, TNF $\alpha$ , and IL-1 $\beta$  mRNA levels peaked at 1 h and then rapidly declined at 3 h, returning to baseline after 8 h (Fig. 2).

Interestingly, TNF $\alpha$ -induced upregulation of inflammatory gene expression in lung was significantly diminished in gp91<sup>phox</sup> <sup>-/-</sup> mice compared to WT mice (Fig. 2). Hence, mRNA levels of E-selectin at 1, 3, and 24 h; IL-1 $\beta$  at 1 h; and VCAM-1, ICAM-1, TNF $\alpha$ , and IL-6 at 3 h were significantly lower in gp91<sup>phox</sup> <sup>-/-</sup> mice compared to WT mice (*P* < 0.05, *n* = 4 or 5). These data indicate that genetic deficiency of gp91<sup>phox</sup> decreases TNF $\alpha$ -induced acute inflammatory responses in the lungs, consistent with the notion that NOX-ROS play an important role in this process.

Exposing WT animals to TNF $\alpha$  for 3 h strongly up-regulated expression of all inflammatory genes measured in all tissues examined (Table 1). Compared to WT mice, inflammatory gene expression was significantly lower in the lungs of gp91<sup>phox</sup> <sup>-/-</sup> and p47<sup>phox</sup> <sup>-/-</sup> mice (Figs. 2 and 3) but, interestingly, not any other tissues (Table 1). Hence, in gp91<sup>phox</sup> <sup>-/-</sup> mice, lung mRNA levels of E-selectin, VCAM-1, ICAM-1, IL-1 $\beta$ , IL-6, and TNF $\alpha$  were diminished by 72, 63, 61, 35, 57, and 51%, respectively (*P* < 0.05, *n* = 4 or 5) (Fig. 2). Similar results were observed in lung tissue of p47<sup>phox</sup> <sup>-/-</sup> mice (Fig. 3). However, lack of gp91<sup>phox</sup> or p47<sup>phox</sup> did not diminish TNF $\alpha$ -induced upregulation of these inflammatory genes in any other tissues (Table 1). In contrast, TNF $\alpha$ -induced



**Fig. 1.** Time course of TNF $\alpha$ -induced changes in serum levels of (A) sVCAM-1, (B) sICAM-1, (C) MCP-1, and (D) TNF $\alpha$  in WT and gp91<sup>phox</sup> <sup>-/-</sup> mice. Wild-type and gp91<sup>phox</sup> <sup>-/-</sup> mice were injected ip with HBSS (0-time control) or TNF $\alpha$  (50  $\mu$ g/kg bw) as described under Materials and methods. At the indicated time points after TNF $\alpha$  injection, the animals were sacrificed and blood was collected. Serum sVCAM-1, sICAM-1, MCP-1, and TNF $\alpha$  were measured by ELISA. Data shown are mean values  $\pm$  SEM of four or five animals. No statistically significant differences between WT and gp91<sup>phox</sup> <sup>-/-</sup> mice were observed.



**Fig. 2.** Time course of TNF $\alpha$ -induced changes in mRNA levels of inflammatory mediators in the lungs of WT and gp91<sup>phox</sup> <sup>-/-</sup> mice. Wild-type and gp91<sup>phox</sup> <sup>-/-</sup> mice were injected ip with HBSS (0-time control) or TNF $\alpha$  (50  $\mu$ g/kg bw) as described under Materials and methods. At the indicated time points after TNF $\alpha$  injection, the animals were sacrificed and total RNA was isolated from the lungs. Real-time quantitative PCR analysis was performed for VCAM-1, ICAM-1, E-selectin, TNF $\alpha$ , IL-1 $\beta$ , IL-6, and GAPDH mRNA. Data are expressed as a percentage of GAPDH, used as the internal control gene, and are presented as mean values  $\pm$  SEM of four or five animals. \* $P$  < 0.05 compared to WT mice.

IL-6 and TNF $\alpha$  gene expression seemed to be further enhanced in the liver of gp91<sup>phox</sup> <sup>-/-</sup> mice (Table 1A).

*Genetic deficiency of gp91<sup>phox</sup> or p47<sup>phox</sup> attenuates TNF $\alpha$ -induced NF- $\kappa$ B activation in the lungs but not other tissues*

It is well documented that the redox-sensitive transcription factors NF- $\kappa$ B and AP-1 play critical roles in TNF $\alpha$ -induced upregulation of many inflammatory genes. We observed that TNF $\alpha$  treatment increased

NF- $\kappa$ B-DNA binding activity in a time-dependent manner in the lungs of WT mice. NF- $\kappa$ B binding activity increased substantially 1 h after TNF $\alpha$  injection, peaked at 3 h, and returned to baseline after 8 h (Fig. 4A). Interestingly, TNF $\alpha$ -induced NF- $\kappa$ B activation was significantly lower at both the 1- and the 3-h time points (by 43 and 57%, respectively) in the lungs of gp91<sup>phox</sup> <sup>-/-</sup> mice compared to WT mice ( $P$  < 0.05,  $n$  = 4 or 5; Figs. 4A and B). Treatment of WT mice with TNF $\alpha$  for 3 h also strongly induced NF- $\kappa$ B activity in various other tissues (Figs. 4B and C). However, TNF $\alpha$ -induced NF- $\kappa$ B activation was not significantly lower in

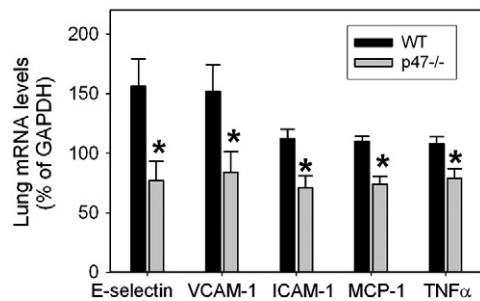
**Table 1**  
Genetic deficiency of gp91<sup>phox</sup> or p47<sup>phox</sup> does not diminish TNF $\alpha$ -induced inflammatory gene expression in nonlung tissues.

		Heart	Liver	Kidney	Small Intestine	Spleen
<b>(A) gp91<sup>phox</sup> <sup>-/-</sup></b>						
E-selectin	WT	208 $\pm$ 27	75 $\pm$ 14	373 $\pm$ 106	384 $\pm$ 149	652 $\pm$ 209
	gp91 <sup>-/-</sup>	171 $\pm$ 31	80 $\pm$ 12	406 $\pm$ 56	303 $\pm$ 52	548 $\pm$ 68
VCAM-1	WT	122 $\pm$ 8	108 $\pm$ 5	87 $\pm$ 17	173 $\pm$ 31	140 $\pm$ 14
	gp91 <sup>-/-</sup>	110 $\pm$ 16	119 $\pm$ 8	92 $\pm$ 9	157 $\pm$ 25	139 $\pm$ 12
ICAM-1	WT	110 $\pm$ 6	84 $\pm$ 6	80 $\pm$ 23	225 $\pm$ 55	165 $\pm$ 32
	gp91 <sup>-/-</sup>	96 $\pm$ 11	97 $\pm$ 8	70 $\pm$ 8	187 $\pm$ 29	193 $\pm$ 15
IL-6	WT	252 $\pm$ 58	136 $\pm$ 16	41 $\pm$ 18	180 $\pm$ 48	212 $\pm$ 60
	gp91 <sup>-/-</sup>	228 $\pm$ 26	345 $\pm$ 43*	32 $\pm$ 9	191 $\pm$ 51	216 $\pm$ 29
MCP-1	WT	185 $\pm$ 25	90 $\pm$ 14	71 $\pm$ 22	315 $\pm$ 101	464 $\pm$ 127
	gp91 <sup>-/-</sup>	162 $\pm$ 29	129 $\pm$ 16	84 $\pm$ 9	386 $\pm$ 81	448 $\pm$ 56
TNF $\alpha$	WT	170 $\pm$ 21	97 $\pm$ 10	71 $\pm$ 18	385 $\pm$ 173	223 $\pm$ 43
	gp91 <sup>-/-</sup>	152 $\pm$ 25	160 $\pm$ 13*	67 $\pm$ 8	128 $\pm$ 18	163 $\pm$ 21
<b>(B) p47<sup>phox</sup> <sup>-/-</sup></b>						
VCAM-1	WT	139 $\pm$ 21		131 $\pm$ 13	107 $\pm$ 6	99 $\pm$ 3
	p47 <sup>-/-</sup>	114 $\pm$ 22		122 $\pm$ 19	123 $\pm$ 11	103 $\pm$ 9
ICAM-1	WT	110 $\pm$ 16		105 $\pm$ 4	107 $\pm$ 8	113 $\pm$ 9
	p47 <sup>-/-</sup>	110 $\pm$ 17		86 $\pm$ 16	118 $\pm$ 15	143 $\pm$ 59
MCP-1	WT	162 $\pm$ 49		130 $\pm$ 14	109 $\pm$ 7	123 $\pm$ 8
	p47 <sup>-/-</sup>	151 $\pm$ 50		134 $\pm$ 23	141 $\pm$ 19	106 $\pm$ 12
TNF $\alpha$	WT	206 $\pm$ 39		113 $\pm$ 17	125 $\pm$ 16	106 $\pm$ 5
	p47 <sup>-/-</sup>	201 $\pm$ 36		99 $\pm$ 21	200 $\pm$ 39	113 $\pm$ 7

Wild-type, gp91<sup>phox</sup> <sup>-/-</sup> (A), and p47<sup>phox</sup> <sup>-/-</sup> (B) mice were injected ip with HBSS (control) or TNF $\alpha$  (50  $\mu$ g/kg bw) as described under Materials and methods. Three hours after injection, the animals were sacrificed and total RNA was isolated. Real-time quantitative PCR analysis was performed for E-selectin, VCAM-1, ICAM-1, IL-6, MCP-1, TNF $\alpha$ , and GAPDH mRNA. Data shown are only from TNF $\alpha$ -treated groups, expressed as a percentage of GAPDH, used as the internal control gene, and presented as mean values  $\pm$  SEM of four or five animals.

\*  $P$  < 0.05 compared to WT mice.





**Fig. 3.** TNF $\alpha$ -induced changes in mRNA levels of inflammatory mediators in the lungs of WT and p47<sup>phox</sup><sup>-/-</sup> mice. Wild-type and p47<sup>phox</sup><sup>-/-</sup> mice were injected ip with HBSS (control) or TNF $\alpha$  (50  $\mu$ g/kg bw) as described under Materials and methods. Three hours after injection, the animals were sacrificed and total RNA was isolated from the lungs. Real-time quantitative PCR analysis was performed for E-selectin, VCAM-1, ICAM-1, MCP-1, TNF $\alpha$ , and GAPDH mRNA. Data shown are from TNF $\alpha$ -treated groups, expressed as a percentage of GAPDH, used as the internal control gene, and presented as mean values  $\pm$  SEM of five animals. \* $P$ <0.05 compared to WT mice.

nonlung tissues of gp91<sup>phox</sup><sup>-/-</sup> mice (Fig. 4B), as well as p47<sup>phox</sup><sup>-/-</sup> mice (Fig. 4C), compared to WT mice ( $P$ <0.05,  $n$ =4 or 5). Lack of gp91<sup>phox</sup> and p47<sup>phox</sup> did not, however, diminish TNF $\alpha$ -induced activation of AP-1 in all tissues examined compared to WT mice (data not shown).

#### Genetic deficiency of gp91<sup>phox</sup> attenuates TNF $\alpha$ -induced acute lung injury

Bronchoalveolar lavage (BAL) fluid was used for assay of total albumin concentration and total neutrophil count as independent assessments of acute lung injury and alveolar inflammation. The TNF $\alpha$ -induced increase in albumin concentration in BAL fluid was significantly less in gp91<sup>phox</sup><sup>-/-</sup> mice compared to WT mice (231  $\pm$  23  $\mu$ g/ml vs 306  $\pm$  20  $\mu$ g/ml,  $P$ <0.05,  $n$ =5). In contrast, a significant increase in lavage neutrophil count was observed in TNF $\alpha$ -treated gp91<sup>phox</sup><sup>-/-</sup> mice compared to WT mice (21.2  $\pm$  3.3  $\times 10^4$  cells vs 7.4  $\pm$  0.9  $\times 10^4$  cells,  $P$ <0.05,  $n$ =5), whereas there was no significant difference between HBSS-treated (control) gp91<sup>phox</sup><sup>-/-</sup> mice and WT mice (4.6  $\pm$  1.0  $\times 10^4$  cells vs 4.4  $\pm$  0.3  $\times 10^4$  cells,  $n$ =5).

#### Genetic deficiency of gp91<sup>phox</sup> or p47<sup>phox</sup> does not affect TNF $\alpha$ -induced neutrophil infiltration into the lungs

Because polymorphonuclear neutrophils (PMNs) and other phagocytic cells, such as monocyte-macrophages, play critical roles in acute inflammation and tissue injury, the specific enzyme marker myeloperoxidase was measured in various tissues after TNF $\alpha$  challenge. As shown in Fig. 5A, TNF $\alpha$  treatment induced time-dependent upregulation of MPO gene expression in the lungs of both gp91<sup>phox</sup><sup>-/-</sup> and WT mice,

which peaked at 3 h and remained elevated for up to 8 h and then declined after 24 h. MPO mRNA levels also increased in other tissues, such as heart, liver, and kidney, albeit to a much lesser degree than in lung (Figs. 5B and C). Interestingly, TNF $\alpha$ -induced changes in MPO gene expression were indistinguishable between gp91<sup>phox</sup><sup>-/-</sup>, p47<sup>phox</sup><sup>-/-</sup>, and WT mice (Figs. 5A–C).

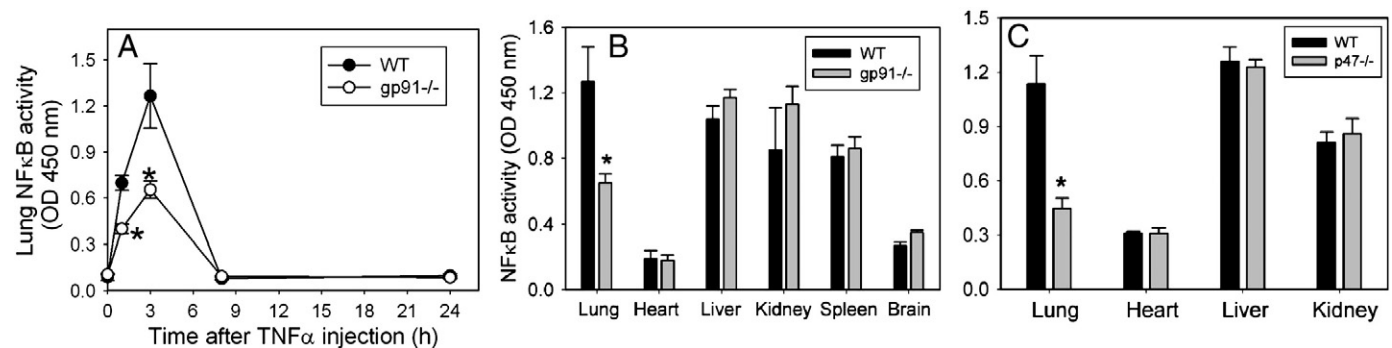
Consistent with increased MPO message levels, MPO protein levels (Fig. 5D) and activity (Fig. 5E) in lung also were significantly increased 8 h after TNF $\alpha$  treatment and indistinguishable between gp91<sup>phox</sup><sup>-/-</sup> and WT mice. Furthermore, morphological analysis showed significantly increased numbers of neutrophils in the lungs of gp91<sup>phox</sup><sup>-/-</sup> mice compared to WT mice ( $P$ <0.05; Figs. 6A, B, and E). TNF $\alpha$  treatment further markedly induced accumulation of abundant interstitial neutrophils in both gp91<sup>phox</sup><sup>-/-</sup> and WT mice; however, there was no significant difference between these two groups of animals (Figs. 6C, D, and E). These data indicate that the lungs are the main target for activated PMNs, and genetic deficiency of phagocytic NADPH oxidase does not impair PMN chemotaxis during TNF $\alpha$ -induced acute inflammation.

## Discussion

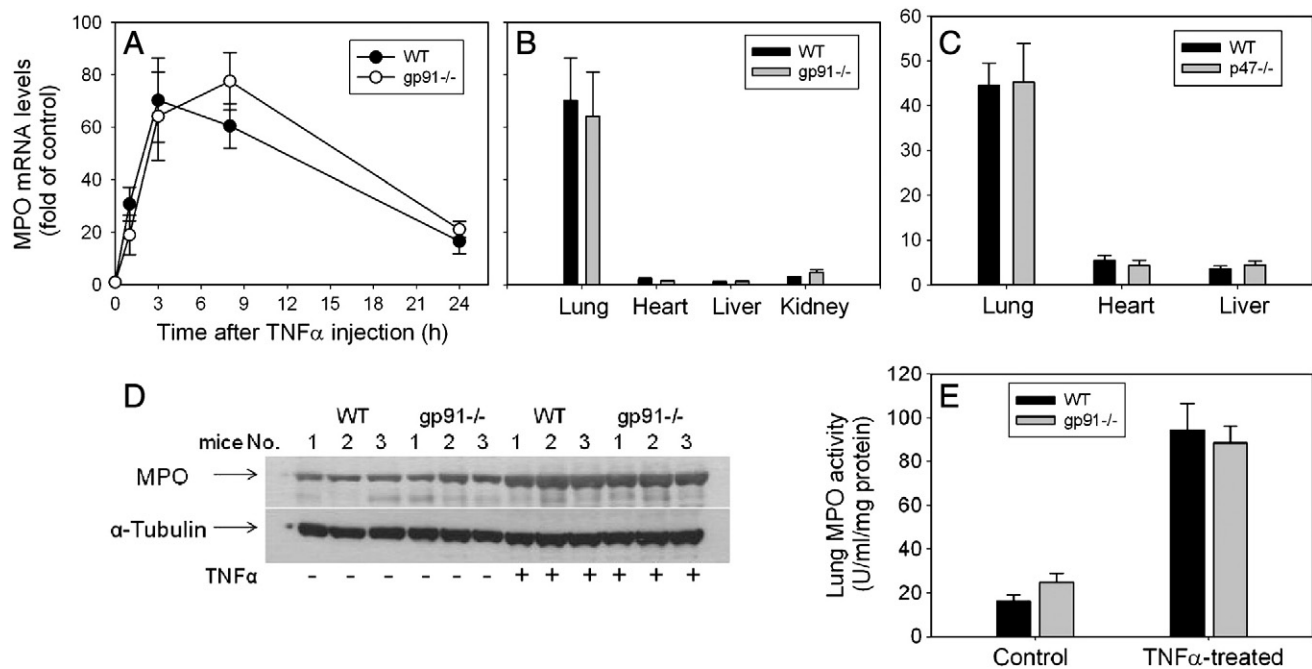
Increased ROS production and oxidative stress contribute to cellular and tissue injury during the development of inflammatory diseases [1,5,10,29,30]. Evidence also suggests that ROS modulate a wide variety of cell-signaling processes [1,5,6]. Yet, the relationship between oxidative stress and the pathology of inflammatory diseases is not fully understood, largely because of limited understanding of the mechanisms by which ROS function as signals in physiological or pathophysiological settings. Recent studies, though still controversial, have suggested that NOX-ROS contribute to oxidative stress and may serve as cell signaling molecules enhancing acute inflammatory responses and tissue injury [1,3,4,14–21]. However, the role of NOX-ROS in TNF $\alpha$ -induced cell signaling and acute inflammatory responses in vivo is less clear.

In this study, we found that genetic deficiency of gp91<sup>phox</sup> or p47<sup>phox</sup> significantly attenuated TNF $\alpha$ -induced NF- $\kappa$ B activation, upregulation of inflammatory gene expression, and acute tissue injury in the lungs but not other tissues of mice. These effects were associated with higher levels of leukocyte infiltration into the lungs compared to other tissues. Therefore, our data provide new evidence that both NOX-ROS-dependent and -independent pathways are involved in TNF $\alpha$  signaling, and especially phagocytic NOX-ROS play an important role in TNF $\alpha$ -induced acute inflammatory responses in the lungs.

It is now widely accepted that increased ROS production during acute lung inflammation largely originates from phagocytic NOX2-expressing cells within the respiratory tract, including alveolar macrophages and other resident or infiltrating inflammatory-immune cells,



**Fig. 4.** TNF $\alpha$ -induced NF- $\kappa$ B activation in the lungs and other tissues of WT, gp91<sup>phox</sup><sup>-/-</sup>, and p47<sup>phox</sup><sup>-/-</sup> mice. Wild-type and gp91<sup>phox</sup><sup>-/-</sup> or p47<sup>phox</sup><sup>-/-</sup> mice were injected ip with HBSS (control) or TNF $\alpha$  (50  $\mu$ g/kg bw) as described under Materials and methods. (A) At the indicated time points or (B and C) 3 h after TNF $\alpha$  injection, the animals were sacrificed and nuclear extracts were isolated. NF- $\kappa$ B (p65)-DNA binding activity was quantified by ELISA. Data shown are from the TNF $\alpha$ -treated groups and presented as mean values  $\pm$  SEM of four or five animals. \* $P$ <0.05 compared to WT mice.



**Fig. 5.** TNF $\alpha$ -induced changes in myeloperoxidase mRNA and protein levels and enzyme activity in the lungs and other tissues of WT, gp91<sup>phox</sup><sup>-/-</sup>, and p47<sup>phox</sup><sup>-/-</sup> mice. Wild-type, gp91<sup>phox</sup><sup>-/-</sup>, and p47<sup>phox</sup><sup>-/-</sup> mice were injected ip with HBSS (0-time control) or TNF $\alpha$  (50  $\mu$ g/kg bw) as described under Materials and methods. (A) At the indicated time points or (B and C) 3 h after TNF $\alpha$  injection, the animals were sacrificed and total RNA was isolated from lung (A) and other tissues (B and C). Real-time quantitative PCR analysis was performed for MPO and GAPDH mRNA. After normalization to GAPDH, the results for the MPO gene were expressed as fold of HBSS-treated control. For (D), 8 h after TNF $\alpha$  injection, the animals were sacrificed and lung homogenates were prepared. MPO protein was analyzed by Western blot as described under Material and methods. Equal sample loading was determined by quantitation of protein as well as reprobing the membranes for  $\alpha$ -tubulin. For (E), 8 h after TNF $\alpha$  injection, the animals were sacrificed and lung homogenates were prepared and MPO activity was assayed as described under Materials and methods. Data shown are mean values  $\pm$  SEM of four or five animals.

including neutrophils, eosinophils, and lymphocytes. However, direct evidence that NOX2 activation contributes to respiratory disease pathology is sparse. Although NOX2 in phagocytic cells primarily functions in host defense by generating antimicrobial ROS, activation of NOX2 in neutrophils was also found to regulate intracellular signaling pathways that modulate neutrophil [29] and endothelial [14] inflammatory gene expression via NF- $\kappa$ B activation. Similarly, stimulation of macrophage NOX2, which produces markedly fewer ROS compared to neutrophils, has been linked to regulation of NF- $\kappa$ B and AP-1 signaling and production of proinflammatory cytokines [30]. In fact, accumulation of infiltrating neutrophils is a key event in the onset and development of inflammation and resultant host tissue injury in the lungs.

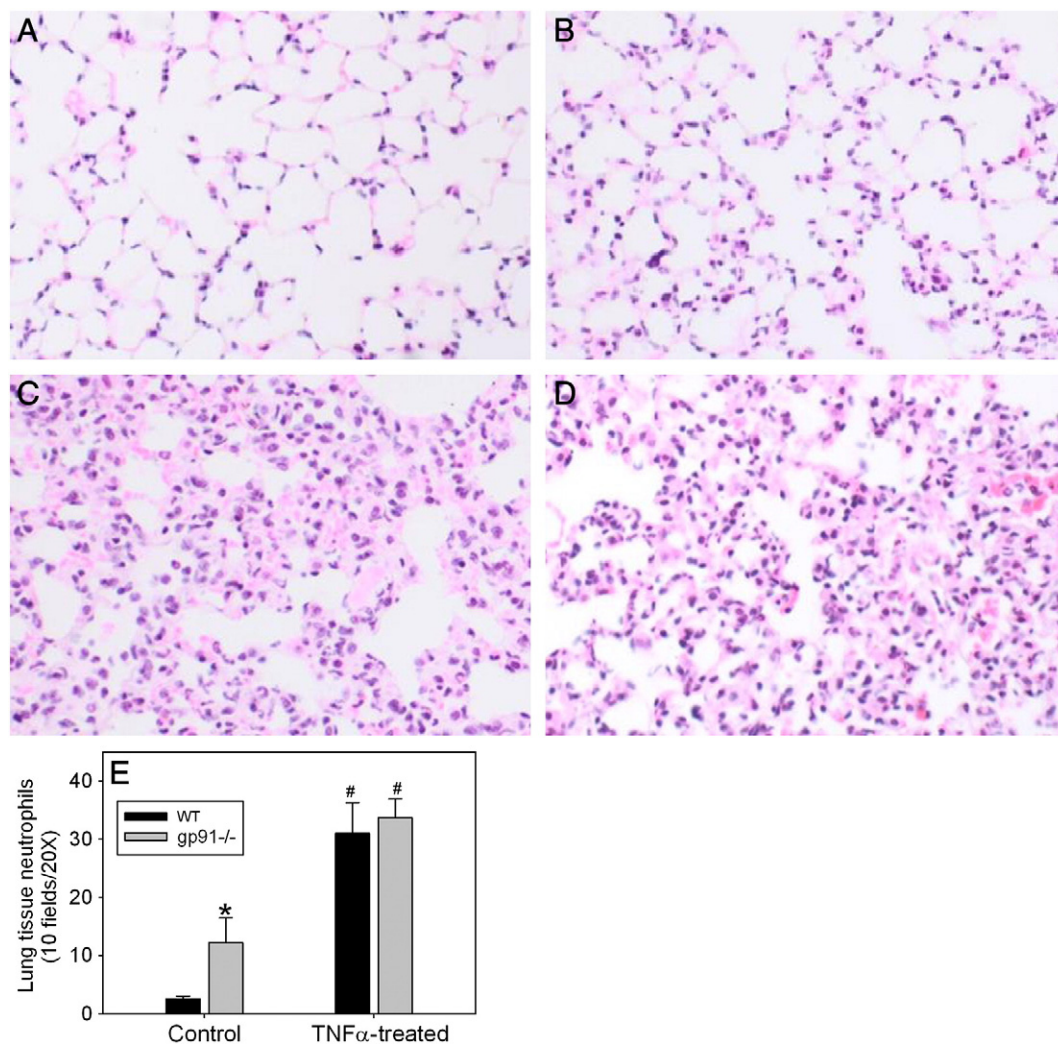
Our data showed that treatment of animals with TNF $\alpha$  strongly increased gene expression, protein levels, and enzymatic activity of MPO, a biomarker of neutrophils, in the lungs but not other tissues, such as heart, liver, and kidney. Furthermore, TNF $\alpha$ -induced increases in lung MPO and accumulation of interstitial neutrophils in the lungs were indistinguishable between NADPH oxidase knockout mice and WT mice. However, deficiency of NOX2 or p47<sup>phox</sup> significantly limited TNF $\alpha$ -induced activation of NF- $\kappa$ B and expression of inflammatory genes. These data indicate that lung is the main target tissue for activated neutrophils during the acute phase of inflammation, and phagocytic NADPH oxidase critically contributes to TNF $\alpha$ -induced inflammatory responses in the lungs. These findings are consistent with previous studies showing that neutrophil NADPH oxidase plays a significant role in the mechanism of TNF $\alpha$ -induced NF- $\kappa$ B activation and ICAM-1 expression in the lungs of p47<sup>phox</sup><sup>-/-</sup> and WT mice [14].

Acute lung injury is characterized clinically by derangements in lung vascular permeability, which is a cardinal feature of inflammation and the initial cause of the profound physiological abnormalities observed in patients with acute lung injury [31]. In this study we found in our murine models that deficiency of NOX2 significantly attenuated the TNF $\alpha$ -induced increase in total albumin concentration in BAL fluid, which reflects leakage of protein into the alveolar space across both the

vascular and the epithelial barriers. The cumulative effects of NOX2 knockout on TNF $\alpha$ -induced lung injury could account for both a decrease in vascular leakage and a reduction in inflammation characterized by reduced lung NF- $\kappa$ B activation and inflammatory gene expression, despite the fact that lung interstitial neutrophil counts were similar between TNF $\alpha$ -treated NOX2 knockout and WT mice. These data indicate that neutrophils lacking a functional NADPH oxidase have normal chemotactic activity but a reduced ability to mount an inflammatory response to TNF $\alpha$  in the lungs, suggesting that phagocytic NADPH oxidase critically contributes to TNF $\alpha$ -induced acute lung injury.

Although it is known that ROS affect a number of signaling pathways by activating or inactivating specific protein kinases and phosphatases, respectively [1], the signaling molecules specifically targeted by ROS in the NF- $\kappa$ B pathway have not been unequivocally identified. Whereas some studies have found that ROS act as second messengers and are involved in TNF $\alpha$  signaling and NF- $\kappa$ B activation [7–12,14], others have not found such evidence [32,34] or even inhibition of NF- $\kappa$ B activation by hydrogen peroxide [33]. It is likely that ROS-mediated NF- $\kappa$ B activation is restricted to certain cell types and incubation conditions, such as the types and concentrations of ROS or proinflammatory cytokines used. In addition, most of the evidence for a role of ROS in NF- $\kappa$ B activation is derived from *in vitro* study, and *in vivo* evidence is scant. Our *in vivo* data suggest that TNF $\alpha$ -induced NF- $\kappa$ B activation in lung is indeed enhanced by NOX-ROS (Scheme 1), as it was significantly diminished in gp91<sup>phox</sup><sup>-/-</sup> and p47<sup>phox</sup><sup>-/-</sup> mice compared to WT mice. In contrast, TNF $\alpha$ -induced activation of AP-1, another critical transcription factor for inflammatory gene expression, was not affected by NADPH oxidase deficiency.

Our data further suggest that in nonlung tissues, NOX-ROS do not play a significant role in TNF $\alpha$ -mediated NF- $\kappa$ B activation and inflammatory gene expression. In this NOX-ROS-independent pathway, TNF $\alpha$  binding to the TNF receptor 1 (TNF-R1) triggers a series of intracellular signals causing activation of I $\kappa$ B kinase (IKK), which results



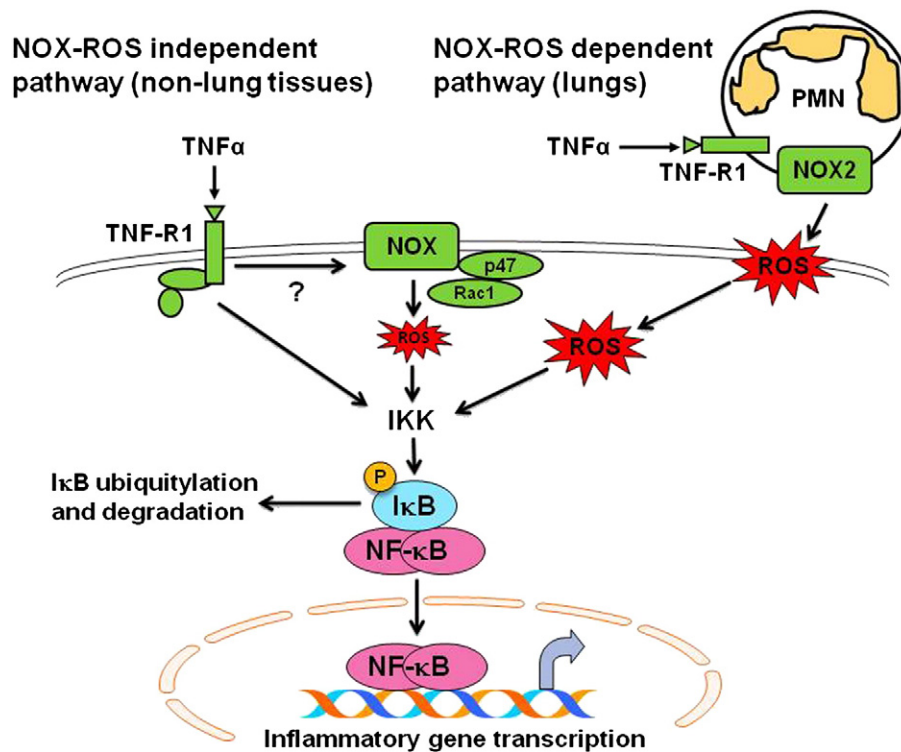
**Fig. 6.** TNF $\alpha$ -induced neutrophil infiltration into the lungs of WT and gp91<sup>phox</sup><sup>-/-</sup> mice. Representative lung tissue sections obtained from WT mice treated with (A) HBSS or (C) TNF $\alpha$  and from gp91<sup>phox</sup><sup>-/-</sup> mice treated with (B) HBSS or (D) TNF $\alpha$  were stained with hematoxylin and eosin (20 $\times$  original magnification), and (E) the infiltrated interstitial neutrophils were counted as described under Materials and methods. Data shown are mean values  $\pm$  SEM of four or five animals. \* $P$ <0.05 compared to WT mice, # $P$ <0.05 compared to HBSS-treated control mice.

in phosphorylation and subsequent ubiquitylation and degradation of I $\kappa$ B and translocation of NF- $\kappa$ B to the nucleus (Scheme 1). In vitro evidence suggests that TNF-R1 stimulation also induces NADPH oxidase activity via protein kinase C-mediated phosphorylation of p47<sup>phox</sup> and activation of Rac1, thereby increasing ROS production and enhancing IKK and NF- $\kappa$ B activation [35,36]. However, our data suggest that this mechanism does not play a significant role in vivo, unless extensive neutrophil infiltration occurs and ROS are generated in large quantities by NOX2, such as in the lungs of TNF $\alpha$ -exposed mice. It is possible that these PMN-derived ROS, in particular hydrogen peroxide—which is relatively long-lived and membrane-permeative—are transmitted into resident tissue cells and serve as signaling molecules activating IKK and NF- $\kappa$ B (Scheme 1). These exogenous ROS generated by NOX2 of infiltrating PMNs seem to be more critical in mediating TNF $\alpha$ -induced inflammatory responses in vivo than ROS signals generated endogenously by NOX in resident macrophages or nonphagocytic cells [14]. Nevertheless, we cannot exclude the possibility that ROS generated from other cellular sources, such as xanthine oxidase, mitochondria, or cytochrome P450, may have played a role in TNF $\alpha$ -induced cell signaling and inflammation in nonlung tissues [5,37].

It should be noted that the results from previous studies for a role of NOX-ROS in lung inflammation in vivo are still controversial. It has been

shown that LPS or *Pseudomonas aeruginosa*-induced NF- $\kappa$ B activation in lung tissue was impaired in p47<sup>phox</sup><sup>-/-</sup> and gp91<sup>phox</sup><sup>-/-</sup> mice [3,4,16], and LPS-induced lung inflammation in Nrf2<sup>-/-</sup> mice was significantly reduced by genetic ablation of gp91<sup>phox</sup> [38], supporting an important role for NOX-ROS in promoting inflammation. However, other investigators found no differences between p47<sup>phox</sup><sup>-/-</sup> and WT mice in LPS-induced lung injury, alveolar damage, and infiltration of neutrophils and monocytes [17] and no amelioration of pulmonary damage in *Pneumocystis*-infected gp91<sup>phox</sup><sup>-/-</sup> mice compared to WT mice [18]. In fact, more recent studies found that genetic deficiency of NADPH oxidase was in many cases associated with enhanced lung inflammation and injury [19–21,39–44], indicating that NADPH oxidase has beneficial functions in limiting inflammatory-immune responses or enhancing their resolution. For example, infection of gp91<sup>phox</sup><sup>-/-</sup> mice with the yeast *Cryptococcus neoformans* or the influenza virus was found to result in increased Th1-skewed inflammation and granuloma formation [42,43]. Deficiency of gp91<sup>phox</sup> was also reported to markedly increase pulmonary cytokine and chemokine expression and neutrophil recruitment in a mouse model of pneumococcal pneumonia [20], and lung levels of proinflammatory mediators, such as keratinocyte chemoattractant, IL-6, MCP-1, and TNF $\alpha$ , were significantly increased in both gp91<sup>phox</sup><sup>-/-</sup> and p47<sup>phox</sup><sup>-/-</sup> mice in response to cigarette smoke





**Scheme 1.** Diagram depicting the NOX-ROS-dependent and NOX-ROS-independent pathways leading to activation of NF-κB. In the NOX-ROS-independent pathway (left), TNFα binds to the TNF receptor 1 (TNF-R1) located on the plasma membrane of target cells, thus activating IκB kinase (IKK). Activated IKK phosphorylates IκB, leading to its ubiquitylation and subsequent degradation. This releases NF-κB and allows its translocation to the nucleus, where it binds to DNA promoter regions and induces inflammatory gene transcription. TNFα binding to TNF-R1 may also induce phosphorylation of p47<sup>phox</sup> and activation of Rac1, which activates NADPH oxidase, thus increasing intracellular production of ROS and enhancing NF-κB activation. The results presented in this paper do not support the notion that NOX-ROS play a significant role in TNFα-induced NF-κB activation in nonlung tissues in vivo (arrow marked “?”). In the NOX-ROS-dependent pathway (right), TNFα binds to TNF-R1 located on the plasma membrane of PMNs, which in turn activates NOX2 and produces extracellular ROS. The ROS from PMNs, in particular hydrogen peroxide, diffuse into target cells and serve as signaling molecules for NF-κB activation, thereby enhancing acute inflammation in the lungs of TNFα-exposed mice.

exposure [19]. Consistent with these data, we have recently shown that genetic deficiency of NADPH oxidase does not diminish, but rather enhances, LPS-induced acute lung inflammatory responses [21]. These results might also be related to clinical observations in patients with the X-linked form of chronic granulomatous disease (gp91<sup>phox</sup> deficiency), who are more prone to infection and present with hyperinflammatory conditions [45–47].

However, in this study we showed that reduced phagocytic NOX-ROS diminished TNFα-induced acute lung inflammatory responses and lung injury, which is opposite to our observations in mice exposed to LPS instead of TNFα [21]. These apparently discrepant results highlight the above notion that the role of ROS in cell signaling and inflammatory gene expression can vary widely depending on, e.g., the proinflammatory cytokine or bacterial agent used and the affected tissue or cell type. More studies are needed to increase our understanding of NOX-ROS-mediated cell signaling, in particular the cellular location of NOX and its association with target cells or proteins. In conclusion, our data suggest that phagocytic NOX-ROS signaling plays a critical role in promoting TNFα-induced, NF-κB-dependent acute inflammatory responses and tissue injury in the lungs, whereas NOX-ROS-independent pathways play a dominant role in nonlung tissues.

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